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Platelet Activation Induced by Porcine Factor VIII (HYATE:C)

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We studied the effects of porcine factor VIII (P-FVIII; Hyate:C) and other coagulation products employed in the management of patients with hemophilia A, on platelet activation in vitro. Exposure of normal resting platelets to P-FVIII resulted in platelet activation, as manifested by increased expression of the platelet surface activation markers CD62, CD63, and activated-GPIIbIIIa, and by activation-induced modulation of expression of normal platelet membrane glycoproteins CD41, CD42, and CD36. In contrast, platelet activation was not observed after exposure of the platelets to human FVIII, FEIBA, recombinant FVIIa, or cryosupernatant plasma. As with thrombin, exposure of platelets to P-FVIII resulted in the generation of platelet microparticles, an effect not seen not with the other products. In contrast to the characteristic reduction in expression in the number of CD42 molecules detected on thrombin-activated platelets, P-FVIII-stimulated platelets showed a small increase in CD42 expression. In contrast to thrombin, P-FVIII did not cause platelet dense granule release. The results indicate that therapeutic P-FVIII activates platelets, likely in ways that are different from the platelet activation seen with thrombin. The observed platelet activation and microparticle generation may provide a ''hypercoagulable'' mechanism for hemostasis with P-FVIII therapy separate from, and additional to, that due to increased circulating FVIII levels. Am. J. Hematol. 57:200–205, 1998. © 1998 Wiley-Liss, Inc.

Key words: platelets; platelet activation; porcine factor VIII; hemophilia

INTRODUCTION

Porcine factor VIII (P-FVIII; Hyate:C) concentrates are used in the management of hemophilic patients with inhibitors to human factor VIII. Early preparations of P-FVIII were associated with a decrease in platelet count post-infusion [1,2]. The thrombocytopenia has been attributed to platelet aggregation, caused by porcine von Willebrand Factor (vWF) present in the P-FVIII product on human platelets [1,3–5]. With more purified P-FVIII products, a mild transient and reversible thrombocytopenia, which appears not to be related to the amount of product infused, has been reported in some cases [1,6,7], suggesting that it may not be due to low levels of contaminating porcine vWF. The restoration of hemostasis with P-FVIII therapy is usually associated with increased measurable functional factor VIII levels in patient plasmas. Some patients, however, demonstrate a good hemostatic response to treatment with P-FVIII concentrate in the absence of increased factor VIII levels [8,9]. We investigated whether P-FVIII activates platelets and thus induces a hemostatic effect due to an acquired ''hypercoagulability'' state separate from that attributable to increased circulating FVIII. The results indicate that, in contrast to other coagulation factor replacement products, P-FVIII activates platelets and generates platelet microparticles.

MATERIALS AND METHODS

Therapeutic Coagulation Products Used

Porcine factor VIII (HYATE:C, Porton Speywood Ltd, Wrexham, UK), intermediate purity FVIII (Hae-

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mate-P, Behringwerke AG, Marburg, Germany), a high purity monoclonal antibody-purified FVIII (Hemofil M, Baxter Corp., Glendale, CA), recombinant FVIII (rFVIII, Kogenate, Bayer Corp., Elkhart, IN), and FEIBA (Immuno AG, Vienna, Austria) were obtained from routine stocks. Recombinant factor VIIa (rFVIIa) was a gift from K. Lawday (Novo Nordisk Canada Inc., Mississauga, Ont.). FEIBA was used at a final concentration of 5 U/ml and rFVIIa at a final concentration of 1 KIU/ml, while the other products were used at final concentrations of 2 U/ml. These concentrations were selected to simulate plasma concentrations in a patient after therapy. Cryoprecipitate and cryosupernatant plasmas were obtained from Toronto Centre of the Canadian Red Cross Society Blood Services and were used at a 1:4 ratio v/v product to blood. ''Aged'' cryoprecipitate was prepared by maintaining freshly thawed cryoprecipitate at 4°C for 96 hr. Purified porcine vWF (126 μg/ml) was a gift from Dr. J.S. Pete Lollar, Emory University, Atlanta, GA.

Preparation and Staining of Platelets Using a Whole Blood Method

Using a 21-gauge needle and light tourniquet, after discarding the first 2 ml of blood, blood from normal healthy laboratory volunteers was drawn into citrated tubes (0.129 M citrate, Becton Dickinson, Rutherford, NJ). The citrated blood was diluted 1:2 with 0.35% bovine serum albumin/phosphate-buffered saline (BSA/PBS), pH 7.4, and supplemented with the synthetic peptide Gly-Pro-Arg-Pro (GPRP, Sigma Chemicals, St. Louis, MO) at a final concentration of 2.5 mM, to prevent thrombin-induced fibrin polymerization and platelet aggregation. As a positive control, human thrombin (Fibrindex, Ortho Diagnostics, Raritan, NJ) was added to the diluted blood sample to a final concentration of 1 U/ml and incubated at 37°C for 10 min. As negative controls (baseline), 0.35% BSA/PBS was added to the diluted blood. To test the effects of the therapeutic coagulation products on platelets, the diluted blood samples were incubated with each product for 30 min at 37°C. Following exposure to agonist, the samples were fixed with an equal volume of 1% (w/v) paraformaldehyde, pH 7.4, for 10 min at 22°C, as previously described [10]. The fixed samples were further diluted 1:5 with 0.35% BSA/PBS. Twenty-five microliters of fixed blood was incubated with saturating concentrations of monoclonal antibody in the dark for 30 min at 22°C [10]. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to CD41 (GPIIbIIIa), CD42 (GPIb), CD36 (GPIV; thrombospondin receptor), CD63 (lysosomal protein), and activated-GPIIbIIIa (measuring the activation-induced conformationally changed CD41) were obtained from Immunotech Coulter, Westbrook, ME. Phycoerythrin (PE)-conjugated anti-CD42 and CD62P (P-selectin) were from Becton Dickinson, San Jose, CA. After incubation, 1 ml of filtered FACSFlow fluid (Becton Dickinson) was added to each tube and the samples were acquired, within 4 hr after staining, using a FACScan flow cytometer (Becton Dickinson), equipped with a 15 mW argon ion laser. Gating on forward light scatter and fluorescence, GPIb- or GPIIbIIIa-positive single platelets were identified, 10,000 events acquired on each sample and analyzed using LYSYS II software (Becton Dickinson). Quantitation of the proportion of cells expressing the marker or, using Simply Cellular beads (Flow Cytometry Standards, San Juan, PR), quantitation of the number of ''molecules per cell'' (antibody binding sites), was performed as described previously [10]. Mepacrine staining for platelet dense granules followed the method described by Gordon et al. [11].

Detection of Platelet Microparticles by Flow Cytometry

Microparticles were distinguished from intact normal platelets on the basis of the characteristic flow cytometric profile of forward light scatter vs. fluorescence with conjugated antiplatelet antibodies, as described by Abrams et al. [12]. The percentage of platelet microparticles was defined as that proportion of all GPIb- or GPIIbIIIa-positive cells that were smaller than 0.8 μ; they showed reduced fluorescence compared to intact platelets, as shown in Figure 1.

Statistical Analysis

Since the basal level of the markers measured differs between different donors, results were ''normalized'' by relating each result to that of the baseline unstimulated resting platelets for that platelet donor, i.e., by calculating the ratio of post:pre exposure to agonist. One-way analysis of variance and paired Student's *t*-test were performed and $P < 0.05$ was regarded as significant.

RESULTS

Activation-Induced Translocation of Intracellular Molecules to Platelet Surface

Table I shows that following exposure of resting platelets to thrombin, there was an increase in platelets expressing surface CD62 (means ± SD, 3 ± 2% prestimulation vs. 96 ± 3% after thrombin; $P < 0.0001$), P-FVIII also increased platelets expressing CD62 (5-fold, $P = 0.0118$), but to a lesser extent ($P < 0.001$) than was observed with thrombin. Hemofil M, recombinant FVIII, recombinant FVIIa, Haemate P, and FEIBA did not increase the number of platelets expressing CD62. Similarly, P-FVIII increased the proportion of platelets expressing CD63 (5-fold), again to a lesser extent ($P <$

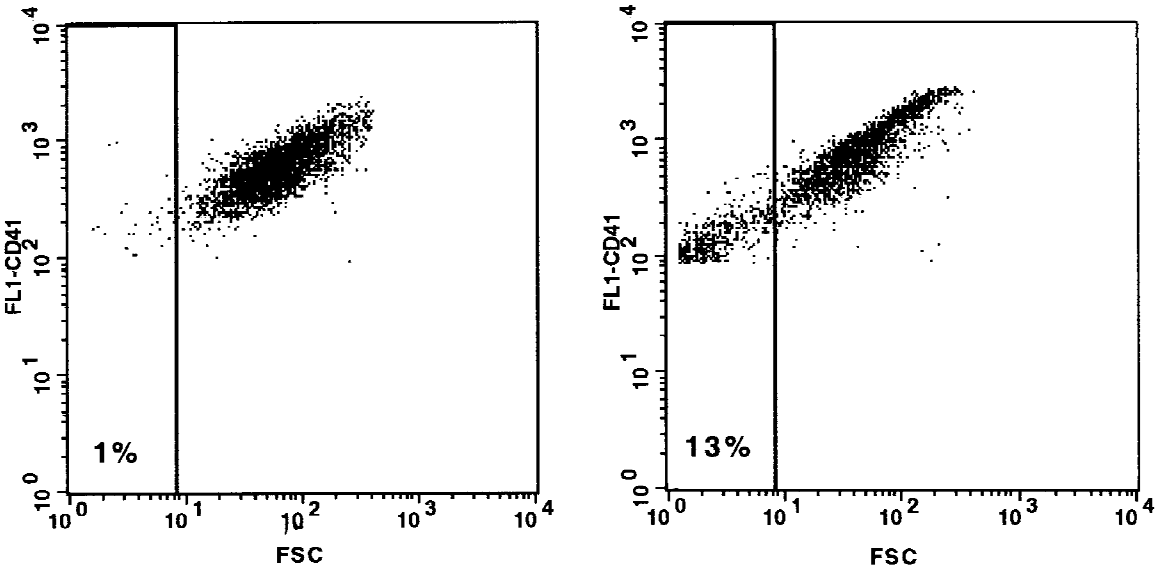

Fig. 1. Flow cytometric dot plot analysis of platelet microparticles, showing two populations of CD41 positive events; the larger cells (right) represent intact platelets, the smaller cells, (left) represent platelet microparticles. FSC = forward light scatter, reflecting the size of the cells; FL1 = fluorescence intensity of bound FITC-labeled anti-CD41.

TABLE I. Proportion of Platelets (mean ± SD) Expressing CD62, CD63, and Activated GPIIbIIIa, Following Incubation With Thrombin and Therapeutic Coagulation Products*

Agonist	N	CD62 (%)	CD63 (%)	Activated GPIIbIIIa (%)
Unstimulated (baseline)	20	3.3 ± 1.8	2.3 ± 1.1	1.3 ± 0.8
Thrombin	20	96 ± 3	71 ± 8	8.8 ± 4.5
P-FVIII	20	15 ± 13	11 ± 6	8.6 ± 9.1
Hemofil M	8	4.8 ± 2.8	2.9 ± 2.1	0.8 ± 0.5
Haemate-P	8	1.6 ± 1.0	1.7 ± 0.4	1.3 ± 0.9
rFVIII	8	3.1 ± 1.2	1.8 ± 0.6	0.6 ± 0.2
FEIBA	8	1.9 ± 1.1	0.7 ± 0.3	3.0 ± 1.2
rFVIIa	8	1.9 ± 0.6	0.9 ± 0.6	0.8 ± 0.6
Cryosupernatant plasma	4	1.4 ± 0.7	0.8 ± 0.3	2.4 ± 0.9
Cryoprecipitate, fresh	8	17 ± 10	4.5 ± 1.4	2.0 ± 1.1
Cryoprecipitate, ''aged''	8	6.0 ± 3.7	2.7 ± 0.5	2.1 ± 1.4

*N = number of experiments.

0.0001) than was seen when thrombin was used as the agonist.

Activation-Induced Conformational Change in Normal Platelet Surface Glycoprotein

Few (1 ± 1%) unstimulated resting platelets showed expression of activated GPIIbIIIa. As shown in Table I, exposure to thrombin in an increase in the proportion of platelets expressing activated GPIIbIIIa. Porcine FVIII also increased activated GPIIbIIIa expression (8-fold, $P = 0.002$), and, in contrast to CD62 and CD63, this was not significantly different from the values observed with thrombin ($P = 0.5954$). None of the other coagulation products tested significantly increased platelet-activated GPIIbIIIa expression.

Activation-Induced Modulation of Normal Platelet Surface Glycoproteins Expression

Expression of CD41, the fibrinogen receptor, is increased upon platelet activation [13]. Resting platelets expressed 49,812 ± 5383 (means ± SD) CD41 molecules per cell (n = 17). As shown in Figure 2, thrombin markedly increased CD41 expression (a 1.74-fold increase, $P < 0.0001$). A significant increase (1.27-fold, $P < 0.0001$) in CD41 expression was also induced P-FVIII, but this was significantly lower than that seen with thrombin ($P < 0.0001$). None of the other coagulation products tested caused an increase in platelet surface CD41 expression.

Platelet surface expression of CD42, the vWF receptor, characteristically is reduced in this system following exposure to thrombin agonist [10,13]. The mean ± SD CD42 on resting platelets was 22,345 ± 4,878 molecules/cell, n = 17. As shown in Figure 2, thrombin exposure reduced the expression of CD42 by a mean of 28% ($P < 0.0001$). In contrast, P-FVIII slightly increased the levels of CD42 expression (mean 9% increase, $P = 0.0014$). None of the other coagulation products significantly altered CD42 expression.

Platelet surface CD36 expression is also characteristically increased after platelet activation. The mean ± SD CD42 on resting platelets was 22,345 ± 4,878 molecules/cell, n = 17. Figure 2 indicates that P-FVIII increased CD36 expression to the same extent as was seen with thrombin. Exposure of platelets to the other coagulation products did not increase CD36 expression.

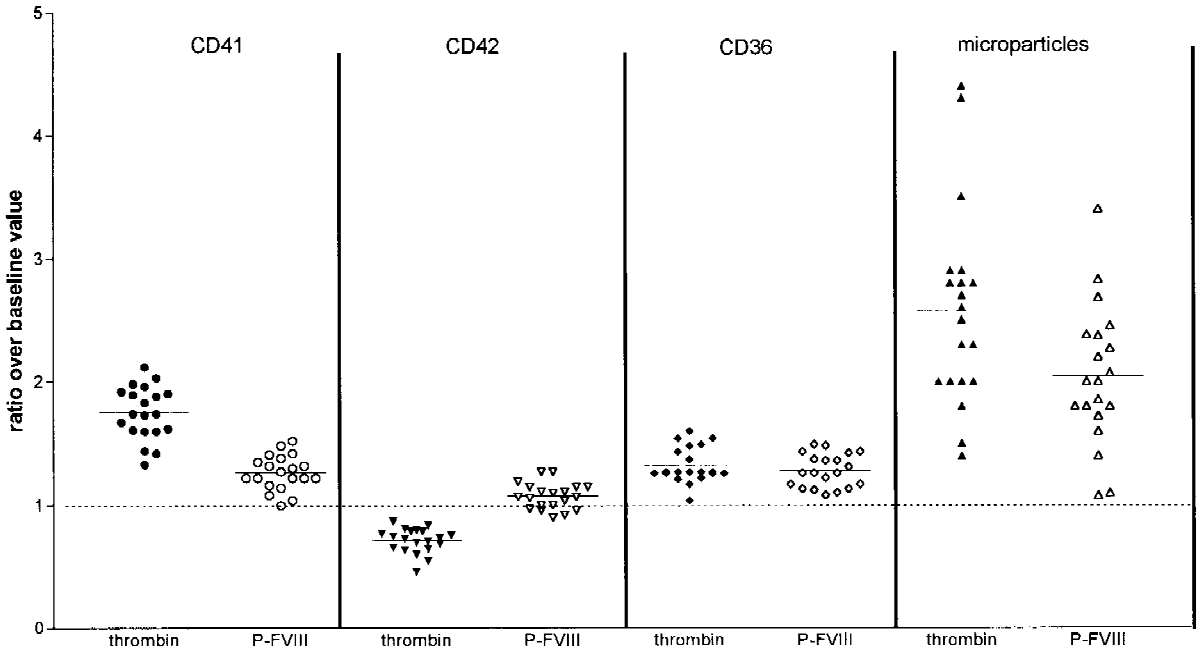

Fig. 2. Ratio of platelet surface expression of CD41, CD42, and CD36 and microparticle generation following exposure to thrombin and to porcine factor VIII compared to levels on basal (unstimulated) resting platelets.

Analysis of Platelet Dense Granules

In contrast to the characteristic thrombin-induced reduction in platelet dense granules, none of the coagulation products tested affected platelet dense granule staining (data not shown).

Microparticle Formation

Platelet activation may be associated with generation of platelet fragments or microparticles. In the resting platelet samples, 4.0 ± 0.8% of all cells positive for the CD41 were identified as microparticles by their size. While exposure to the other coagulation products did not result in an increase in microparticle generation, as shown in Figure 2, exposure of platelets to either thrombin or P-FVIII significantly increased microparticles (2-fold increase with thrombin and 1.8-fold increase with PFVIII, $P = 0.0002$ and $P < 0.0001$, respectively). The extent of microparticle generation was not different between thrombin and P-FVIII ($P = 0.3293$).

Exposure of Platelets to Cryoprecipitate and Cryosupernatant Plasma

Exposure to cryosupernatant plasma did not result in platelet activation by any of the measurements employed. As shown in Table I, exposure of platelets to cryoprecipitate appeared to increase the number of platelets expressing CD62 (to the same extent [$P = 0.9$] as seen with P-FVIII), and this effect was attenuated with ''aged'' cryoprecipitate (a 2-fold increase over baseline, $P = 0.023$; $P = 0.001$ compared to fresh cryoprecipitate). Cryoprecipitate did not significantly increase the number of platelets expressing CD63 or activated GPIIbIIIa (Table I). While exposure of platelets to cryoprecipitate did not affect CD41, CD42, or CD36 platelet surface expression, the samples incubated with cryoprecipitate appeared to contain the greatest amount of microparticle formation (a 5.2-fold increase over unexposed platelets, $P < 0.0001$) and this effect was less marked with ''aged'' cryoprecipitate (3.3-fold increase, $P = 0.009$, not shown). However, subsequent examination of cryoprecipitate (both fresh and aged) alone, i.e., without incubation with platelets, showed that platelet microparticles were already present in the product, in an amount sufficient to explain the apparent increase observed when incubated with test platelets.

Exposure of Platelets to Porcine vWF

Platelets were also incubated under identical conditions with varying concentrations (serial dilutions from 0.0002 μg/ml to 50 μg/ml) of porcine vWF. Limited studies ($n = 3$) showed that, with the exception of the slight increase in CD42 expression seen with P-FVIII, the porcine vWF yielded results similar to the P-FVIII preparation when used at a concentration of 1–2 μg/ml. No effect on microparticle generation or platelet activation in any of the parameters measured was seen at lower concentrations and little or no effect was seen at higher concentrations other than a progressive blockade of surface CD42 sites with an increasing concentration of porcine vWF.

DISCUSSION

The induction of human platelet aggregation in vitro by P-FVIII was recognized in 1973 [14] and was shown to be due to porcine vWF present in the FVIII concentrates [3–5]. Porcine vWF can bind to platelet CD42 and activate the CD41 receptor on human platelets [4]; the binding of porcine vWF to CD42 in the absence of any inducers, such as ristocetin or botrocetin, results in platelet-platelet interaction, which leads to platelet aggregation. The thrombocytopenia observed with early preparations of P-FVIII was attributed to the platelet aggregation. Newer preparations of P-FVIII (HYATE:C) are more highly purified freeze-dried concentrates of porcine antihemophilic factor, prepared by ion-exchange chromatography with polyelectrolytes to separate and remove porcine vWF [15], and are generally associated with less significant thrombocytopenia post-infusion [6–8].

We examined platelet activation following incubation of normal human platelets with various therapeutic products employed in the management of patients with coagulation deficiencies. Incubation with monoclonal purified FVIII, recombinant FVIII, Haemate-P, FEIBA, recombinant FVIIa, and cryosupernatant plasma, did not result in platelet activation. Incubation with P-FVIII, in contrast, resulted in an increase in platelets expressing CD62, CD63, and activated GPIIbIIIa, increased platelet surface expression of CD36 and CD41. These changes were consistent with those observed with thrombin-induced platelet activation. Fewer platelets expressed activated GPIIbIIIa after exposure to thrombin and P-FVIII than those expressing CD62 and CD63 (Table I); this relates to the requirement for fibrinogen to demonstrate altered GPIIbIIIa conformer, but in the test system employed the plasma is diluted 32-fold, reducing available fibrinogen. Although the same concentrations of thrombin, P-FVIII, and platelets were used throughout, with some markers of platelet activation the magnitude of the activation-induced changes with P-FVIII was lower than seen with thrombin (e.g., CD62, CD63, CD41), but for others (e.g., CD36 and activated GPIIbIIIa) the magnitude of change was comparable. Thrombin caused a decrease in CD42 expression on the platelet surface, whereas P-FVIII caused a slight, but significant, increase. Although we have examined platelet activation in a variety of clinical disorders and under a variety of in vitro conditions, this is the only platelet agonist that caused an increase in platelet surface CD42 expression with platelet activation; the explanation for this observation remains to be determined. The differences in degree of platelet activation with some markers suggest that the mechanism of activation may be different between thrombin and P-FVIII. The observations with porcine vWF suggest that the platelet activation and microparticle generation induced by therapeutic concentrations of P-FVIII (HYATE:C) are due to small amounts of residual porcine vWF in the preparation. Preliminary studies suggest that there is an optimal concentration of porcine vWF to achieve these effects. As discussed below, the effects of residual porcine vWF may be desirable.

The apparent platelet microparticle generation observed following incubation with cryoprecipitate seems to be due to platelet microparticles already present in the cryoprecipitate product; this is consistent with previous observations ([16] and personal communication, Georges Rivard, Montreal, 1996) and may be due to the processing, freezing, and thawing of these products.

Platelet activation has been associated with hypercoagulable and thrombotic states [17,18]. Platelet microparticles are formed when there is strong platelet activation. They contain platelet membrane CD41 and CD42, as well as membrane cytoskeletal proteins [19,20]. Microparticles derived from platelet activation are highly enriched in factor Va and Xa binding sites and exhibit prothrombinase activity [19,21]; they can also bind factors VIIIa and IXa [22,23]. In addition, platelet microparticles adhere to the subendothelium and promote platelet binding [24]; they provide a catalytic surface for production of both factor Xa and thrombin [19,20]. Infusion of microparticles shortens the bleeding time and the activated partial thromboplastin time in thrombocytopenic rabbits [25,26], and Warkentin et al. suggested that generation of procoagulant microparticles could explain the thrombotic complications in patients with heparin-induced thrombocytopenia [27]. Others have suggested that increased circulating platelet microparticles may increase the risk of thromboembolic events in patients with hemolytic uremic syndrome and thrombotic thrombocytopenic purpura [28], transient ischemic attacks, lacunar infarcts, and multi-infarct dementias [29,30].

Although the efficacy of P-FVIII (HYATE:C) generally correlates with the increment in circulating factor VIII achieved, a number of good or fair responses have been observed in the absence of a measurable factor VIII increment [8]. This somewhat surprising observation was confirmed by French investigators [9]. Whether the in vitro observations reported here occur in vivo is currently under investigation. Based on our findings to date, we hypothesise that the platelet activation and microparticle generation following infusion of P-FVIII may induce a hypercoagulable state that could explain the enhanced hemostatic response to P-FVIII observed in some hemophiliacs even in the absence of increased circulating factor VIII levels.

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